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FRACTIONATION OF LIVETIN AND THE MOLECULAR WEIGHTS OF THE α- AND β-COMPONENTS[1]

W. G. MARTIN, J. E. VANDEGAER,[2] AND W. H. COOK

Abstract

Livetin, the major water-soluble protein of hen egg yolk, was found to contain three major components having mobilities of −6.3, −3.8, and −2.1 cm.^{2} sec.$^{-1}$ volt^{-1} at pH 8, μ 0.1, and these have been designated α-, β-, and γ-livetin respectively. The α- and β-livetins were separated and purified electrophoretically after removal of γ-livetin by precipitation from 37% saturated ammonium sulphate or 20% isopropanol. The α-, β-, and mixed livetins resembled pseudoglobulins in solubility but γ-livetin was unstable and this loss of solubility has, so far, prevented its characterization. Molecular weights determined by light scattering, osmotic pressure, and Archibald sedimentation procedure yielded respectively: 8.7, 7.8, and 6.7 × 10^4 for α-livetin, and 4.8, 5.0, and 4.5 × 10^4 for β-livetin. Under suitable conditions of sedimentation and electrophoresis, egg yolk has been shown to contain three components having the same behavior as the three livetins of the water-soluble fraction.

Introduction

The major water-soluble protein obtainable from egg yolk, after extraction with ether and the precipitation of the lipoproteins by dilution, has been termed "livetin" (10, 17). Present electrophoretic evidence (19) indicates that this fraction may contain three and possibly more major components, which have yet to be isolated and characterized. Several minor components have also been reported and others might reasonably be present in this water-soluble fraction (7, 15, 23). The present paper describes the separation of the major water-soluble components, and the determination of the molecular weight of two of them.

A previous paper (22) reported that egg yolk diluted with 5% sodium chloride showed one rising (R) and two sedimenting fractions when examined in an analytical ultracentrifuge. The rising fraction is the lipid-rich protein lipovitellenin (8), probably associated with free lipid since the material was not ether-extracted. The major sedimenting fraction contains lipovitellin (22). Subsequent work has shown that this fraction also contains material more soluble in dilute salt solution than lipovitellin whilst the minor fraction contains water-soluble material.

[1]Manuscript received November 28, 1956.
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada.
Issued as N.R.C. No. 4261.
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Methods

Phosphate buffer of ionic strength 0.1 and pH 7.9 was used as solvent unless otherwise noted.

Specific refractive index increments (dn/dc) were measured on a differential refractometer (3) calibrated by drying aliquots of the solutions and dialyzates to constant weight at 105° C. in an air oven. These values were thereafter used to determine concentration, except for the dilute solutions used for light scattering, where the concentrations were determined from the extinction coefficients at 2800 Å in a Beckman spectrophotometer calibrated against the refractometer.

Nitrogen analyses were made by the Kjeldahl method (16) and phosphorus by the method of King (12). Lipid determination was made by lyophilizing the sample in a volumetric flask, making up to volume with 2:1 chloroform–methanol, shaking, and filtering. An aliquot of the filtrate was evaporated at room temperature and the extracted lipid taken up in anhydrous ether, filtered into a tared weighing bottle, and dried at 105° C. in an air oven.

The molar ratio of tyrosine to tryptophan was determined for the livetins at 0.1% concentration in 0.1 N sodium hydroxide with a Carey recording spectrophotometer between λ = 2700 and 3400 Å (2).

Electrophoretic mobilities were measured with either a Klett or Perkin–Elmer apparatus. Where desired the areas of the peaks were measured from enlarged tracings with an Amsler mechanical integrator.

Osmotic pressures were measured at five concentrations between 0.15 and 1.2%. Equilibrium was established in both directions after about 20 hours. Osmometers (6) with a plastic plug in each sac permitted measurements with about 3 ml. of solution.

Sedimentation coefficients were measured at 25° C. in a Spinco analytical ultracentrifuge and the results are reported as $S^0_{20,w}$. The Archibald method (1, 13) was used to estimate the molecular weight of the isolated livetin components, assuming a partial specific volume ($\bar{V}$) of 0.75. Two determinations were made on each component at concentrations ranging from 0.4 to 1.3% at 12,590 r.p.m. in a standard 12 mm. cell. A mechanical integrator was used to evaluate the refractive index patterns (20).

Light-scattering measurements were made at 90° and λ = 4360 Å on the separated livetin components in a Brice–Speiser light-scattering photometer (4). Solutions used for light scattering were centrifuged for an hour at 40,000 r.p.m. in a Spinco preparative centrifuge, drawn into a dust-free pipette, and then filtered through a glass filter into a small cell. Solvent was also filtered into the cell. Samples were removed from the cell after each measurement for concentration determination and solvent added to provide the desired dilution. The refractive index increments (dn/dc) at λ = 4360 Å (Table I) were used to evaluate K in the equation:

$$K = 2\pi^2 n_0^2 (dn/dc)^2 / N\lambda^4 = 5.78 \times 10^{-7}$$

where n_0 = refractive index of solvent, N = Avogadro's number, and the other quantities have been defined earlier.

Results

Preparation and Fractionation

The preparative steps were done at 4° C. Yolks were obtained from hen eggs about 1 day old. The isolated yolks were rolled on absorbent tissue paper and pierced so that the contents could be poured from the membranes which adhered to the paper. The yolk fluids were diluted with three volumes of distilled water and the precipitated lipovitellin removed by centrifugation.

The supernatant was shaken with an equal volume of ethyl ether in a separatory funnel and allowed to stand for several hours. The lower aqueous layer was then readily separated from the intermediate emulsified lipovitellenin and the ether–lipid layer. The aqueous layer was washed three times with ethyl ether, dialyzed against distilled water, and lyophilized. Only negligible amounts of this product were insoluble at low ionic strengths, indicating freedom from lipoproteins. This insoluble material was removed by centrifugation.

The water-soluble proteins were also prepared by shaking egg yolk with two volumes of water and two volumes of carbon tetrachloride. The resulting emulsion was broken by centrifuging at 30,000 r.p.m. and the aqueous layer at the top decanted for re-extraction, dialyzed against distilled water, and lyophilized. Both isolation procedures yielded similar products.

Electrophoretic examination of this material at 1% concentration in glycine (Fig. 1A) or phosphate buffer revealed three major components. These had mobilities of *ca.* -6.3, -3.8, and -2.1 cm.^{2}sec.$^{-1}$volt^{-1} at pH 8, μ 0.1, and have been designated α-, β-, and γ-livetin respectively. In the analytical ultracentrifuge two components were evident initially (Fig. 1D) but on further sedimentation the slower peak widened (Fig. 1E) and was resolved into two peaks after the fast component had disappeared. Three components are therefore detectable by both procedures. Anticipating results to appear below, the components in the sedimentation diagrams have been identified with those found in the electrophoretic patterns.

γ-Livetin was removed from the mixture by precipitation with isopropanol at 20% v/v at 0° C. or with 37% saturated ammonium sulphate at 4° C. Examination of the supernatant after dialysis and lyophilization showed that γ-livetin was absent from the electrophoretic pattern (Fig. 1B) and the fastest sedimenting component was lacking in the sedimentation patterns (Figs. 1F and 1G). This identifies the latter with γ-livetin. The precipitate was γ-livetin (Figs. 1C, 1H, and 1J) contaminated with the other components. Since the object was to obtain material of sufficient purity for physical examination rather than quantitative yields, this procedure was considered satisfactory for the preparation of the α- and β-livetins, although it resulted in considerable loss of these components in the γ-livetin precipitate.

Attempts to purify γ-livetin have shown it to be less stable than the other livetins. On removal of most of the other proteins, its solubility in water is reduced and an ionic strength of about 0.1 is required to dissolve the material.

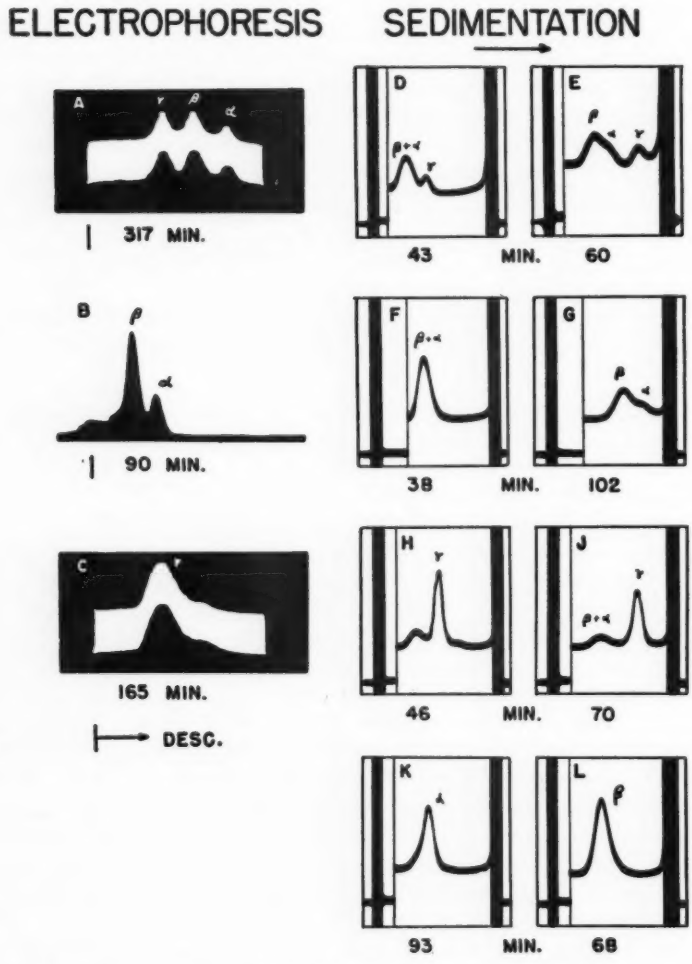

FIG. 1. Electrophoretic (A, B, C) and sedimentation (D–L) patterns of livetin fractions.
Unfractionated livetin: A, buffer glycine pH 9.0, μ 0.3; D, E, solvent 0.2 *M* NaCl.
Supernatant from 37% saturated ammonium sulphate: B, buffer phosphate pH 7.9, μ 0.1; F, G, solvent 0.1 *M* NaCl.
Precipitate from 37% saturated ammonium sulphate: C, buffer phosphate pH 7.9, μ 0.1; H, J, solvent 0.1 *M* NaCl.
Electrophoretically separated livetins: K, α-livetin; L, β-livetin; buffer phosphate pH 7.9, μ 0.1.

A marked loss in solubility occurs at each purification step, on freeze-drying, or even on standing. The sedimentation coefficients observed on the soluble portion of partially purified preparations lie between 7 and 8 $\times 10^{-13}$, indicating that γ-livetin has a much higher molecular weight than the other livetins. Adequate physical measurements cannot be made until this fraction can be purified without loss of solubility.

Precipitation with ammonium sulphate at several concentrations failed to separate the α- and β-components. Half saturation with ammonium sulphate precipitated 83% (by weight) of the mixture from a 1% solution. Sedimentation patterns on both the precipitated and soluble material were similar. The material soluble in half-saturated ammonium sulphate was completely precipitated by 5% trichloracetic acid.

Since differential precipitation showed little promise, electrophoretic separation of the α- and β-livetins on a small scale was made with a Kekwick cell (11). A few hundred milligrams of the separated components were prepared, dialyzed against distilled water, and lyophilized. The sedimentation diagrams of these electrophoretically purified materials are shown in Figs. 1K and 1L.

If these livetins occur in whole egg yolk, they should be demonstrable under suitable conditions. Sedimentation in 5% salt solutions showed only the two sedimenting components (S1 and S2) previously described (22). In this solvent S1 was homogeneous electrophoretically. Sedimentation and electrophoretic analyses were therefore undertaken in several other solvents. Diluted egg yolk was used for sedimentation, but for electrophoresis most of the rising fraction was removed by centrifugation to avoid disturbances during electrophoresis caused by local density variations. The electrophoretic pattern in glycine buffer (pH 9.0, μ 0.3) and sedimentation pattern in 1% ammonium carbonate solution appear in Fig. 2.

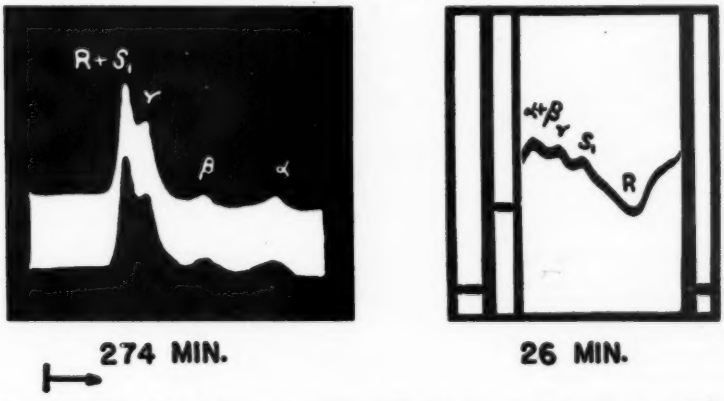

FIG. 2A (left). Electrophoretic pattern of diluted egg yolk, less most of the lipovitellenin fraction, buffer glycine pH 9, μ 0.3.
FIG. 2B (right). Sedimentation pattern of diluted egg yolk, solvent 1% ammonium carbonate.

In the electrophoretic pattern (Fig. 2A) the peak of slowest mobility is a composite of the two lipoproteins. The other peaks are designated α, β, and γ, since their mobilities are similar to those of the α-, β-, and γ-livetins of the water-soluble protein. In sedimentation (Fig. 2B) the rapidly sedimenting lipovitellin is followed by γ-livetin and the unresolved α- and β-livetin peak. The identification is based upon the similarity of the sedimentation coefficients to those of isolated lipovitellin and the water-soluble livetins.

Composition of α- and β-Livetin

The limited analytical results are summarized at the top of Table I. These show that α- and β-livetin are similar in gross composition and refractive index increments but differ in their molar tyrosine/tryptophan ratio. The phosphorus content is low and the lipid content negligible.

Molecular Weight of α- and β-Livetin

The sedimentation coefficients, extrapolated to zero concentration, and the slope of the s versus c line (Table I) give quantitative confirmation of the results in Fig. 1 and show that concentration dependence is small. These results also distinguish α- and β-livetin, since the former, having the higher electrophoretic mobility, also has the higher sedimentation rate.

TABLE I

SUMMARY OF CHEMICAL AND PHYSICAL MEASUREMENTS ON α- AND β-LIVETIN

Quantity	α-Livetin	β-Livetin	Unseparated* α- and β-livetin mixture
Lipid, %	0.1	0.1	0.1
Nitrogen, %	14.3	14.3	14.3
Phosphorus, %	—	—	0.12
Nitrogen, lipid free, %	—	—	14.3
Phosphorus, lipid free, %	—	—	0.12
Molar tyrosine/tryptophan ratio	6.4	2.4	—
dn/dc, $\times 10^3$ g./dl.			
at λ = 5890 Å	1.81	1.81	—
at λ = 4360 Å	1.89	1.89	—
$E_0^{1\%}$ at λ = 2800 Å	7.38	9.44	—
Mobility, cm.^{2} sec.$^{-1}$ volt^{-1}	−6.3	−3.8	—
$S^0_{20,w}$, Svedbergs	4.38	3.00	3.38
ds/dc, Sved./1%	−0.54	−0.22	−0.38
Molecular weight $\times 10^{-4}$			
Light scattering (M_w)	8.7 ± 0.6	4.8 ± 0.1	—
Osmotic pressure (M_n)	7.8 ± 0.8	5.0 ± 0.4	—
Archibald sed. proc.† (M_w)	6.7	4.5	—

*α:β: :2:5 from electrophoretic pattern.
†Assumed $\bar{V}$ = 0.75.

The plot of KC/R_{90} versus C, where R_{90} is the reduced intensity of the light scattered at 90° and C is the protein concentration in g./ml., derived from the light-scattering experiments, is shown for both components in Fig. 3A.

The plot of π/CRT versus C, where π is the pressure in centimeters of water at 4° C., C is the concentration in g./100 ml., R is the gas constant, and T is absolute temperature, from the osmotic pressure experiments, is shown in Fig. 3B. The lines were fitted by least squares and the molecular weights, shown in Table I, were computed from the reciprocals of the intercepts. The standard errors of the intercepts were used to estimate the standard errors of the molecular weights.

The results obtained by light scattering, osmotic pressure, and sedimentation yielded values of 8.7, 7.8, and 6.7 × 10^4 respectively, as the molecular weights of α-livetin, while those for β-livetin were 4.8, 5.0, and 4.5 by the three methods.

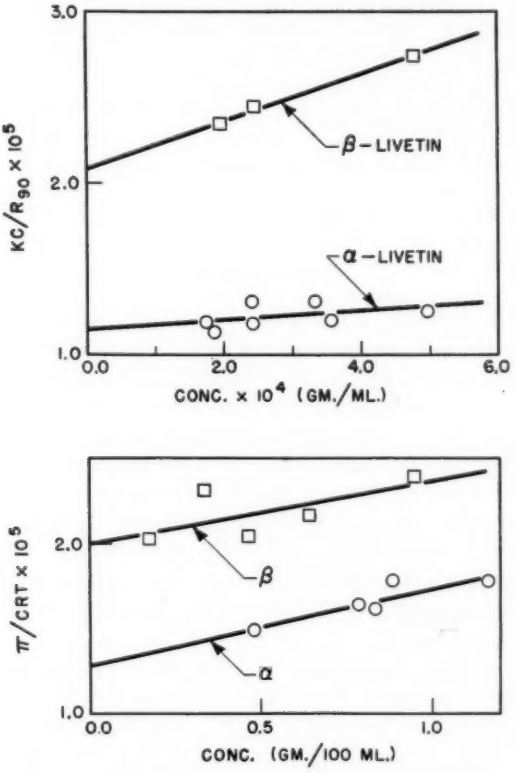

FIG. 3A (top). Light scattering at 90° of solutions of α- and β-livetin.
FIG. 3B (bottom). Osmotic pressure of α- and β-livetin solutions.

The small amount of material available made concentration determinations difficult and this doubtless contributes to the experimental error. Inherently the results obtained by the Archibald method are subject to the greatest uncertainty. First, the partial specific volume was assumed; second, the molecular weight could be determined only at the meniscus and this would give minimum values for the molecular weight if the material were not homogeneous; and finally, an objective estimate of the error was impossible from the available results obtained by this method.

Clearly, the experimental error within each method is about $\pm 10\%$. The differences in molecular weight between methods, which include number average (M_n osmotic pressure) and weight average (M_w light scattering) procedures, do not significantly exceed these limits of error for β-livetin. This indicates that this component is reasonably homogeneous and free from the apparently much larger γ-livetin. The low mobility of γ-livetin makes it the most likely contaminant where electrophoretic separation is the final step in purification.

The differences in molecular weight for α-livetin do not exceed the experimental errors of the methods employed. Its higher mobility should ensure complete separation from γ-livetin electrophoretically. The low phosphorus content of the mixed α- and β-livetins shows that even if it were contributed entirely by phosvitin (10% P), there was a negligible amount of this low molecular weight material present. If α-livetin is heterogeneous and this is due to an impurity, some minor component in the water-soluble fraction would seem to be the most likely contaminant.

Discussion

Kay and Marshall (10) found that livetin was not a lipoprotein but a pseudoglobulin of low phosphorus content. They suspected that livetin was not a homogeneous entity and subsequent electrophoretic analysis (19) has indicated three principal components. In the present work three major components have been found in the water-soluble material and the mixture has the solubility traits of a globulin. Except for a lower nitrogen content, the available analytical results for α- and β-livetin are similar to those given for the mixed livetins (10, 17), which indicates that all components are similar in gross chemical composition. Since the mixture has the properties of livetin, as defined by the original workers (10, 17), the three components, which differ substantially in mobility and molecular weight, have been termed α-, β-, and γ-livetin.

The two recognized lipoproteins and the three livetins indicate at least five components in egg yolk. The results of Clegg *et al.* (5) on ether-extracted egg yolk indicate four components. In this study four components were also found in egg yolk, diluted with suitable solvents, by sedimentation and electrophoretic methods (Fig. 2). Because of the short period required to demonstrate all components, the two lipoproteins are not resolved electro-

phoretically, and the α- and β-livetins are not separable by sedimentation. The five components in Fig. 2 are therefore: floating boundary, lipovitellenin; first sedimenting boundary, lipovitellin; second sedimenting boundary, γ-livetin; third sedimenting boundary, the combined α- and β-livetins which are resolved electrophoretically.

The mixed livetins are reported by Kay and Marshall (10) to represent a fifth to a quarter of the total yolk protein. It is doubtful that their analytical procedure included all the γ-livetin since in the present work it was found that much of this component was always lost with the lipovitellin. While no quantitative estimates were attempted, the refractive index patterns, exemplified in Fig. 2, suggest that the mixed livetins may represent about half, and γ-livetin alone about a quarter, of the protein in egg yolk.

Since this work was completed, an abstract by Ray *et al.* (18) has appeared indicating that five components can be demonstrated in whole egg yolk by the use of solvents not tested in the present study. The results do not appear to be in complete agreement with the present work, however, since Ray *et al.* found two lipoproteins (which may be fractionated further), phosvitin, and two other components. The solvents and method used here did not yield phosvitin in the water-soluble fraction, and as it is part of, or firmly bound to lipovitellin (9), it was not to be expected. Some of the sedimentation coefficients reported by Ray *et al.* likewise differ from those found or indicated by the present work.

The mobilities reported by Shepard and Hottle (19) for the three components in their ether-extracted preparations are similar to those found here for the three livetins. They found that the component of lowest mobility could be precipitated with 18% ethanol. In this study γ-livetin was precipitated by 20% isopropanol or 37% saturated ammonium sulphate. Conalbumin is also precipitated by 20% ethanol, and has been reported (15) to the extent of 4% in the water-soluble fraction of yolk. γ-Livetin is present in much larger proportions and is precipitated below half saturation with ammonium sulphate in which conalbumin is soluble. Clearly, γ-livetin and conalbumin are different entities. Characterization of γ-livetin must await its isolation in pure form and this is under investigation.

The molecular weight of β-livetin is comparable with that of ovalbumin, but the former's mobility, tyrosine–tryptophan ratio and sedimentation coefficient are all lower (14, 21, 23). Ovalbumin has been reported (15) in the water-soluble yolk proteins but in negligible amounts (0.2%) compared with the β-livetin present. Again β-livetin behaves as a pseudoglobulin and is largely precipitated at half-saturated ammonium sulphate in which ovalbumin is soluble.

Although α-livetin also has the solubility behavior of a pseudoglobulin, several of its properties are comparable with those of serum albumins. In fact, Shepard and Hottle (19) reported that their high mobility component and hen serum albumin were similar electrophoretically. The present results

show that α-livetin is similar to serum albumins in sedimentation coefficient, molecular weight, and tyrosine–tryptophan ratio. In spite of the similarities, the solubility behavior indicates that α-livetin is not identical with serum albumin.

Acknowledgments

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SENSITIZATION BY INSULIN TO THE DEXTRAN "ANAPHYLACTOID" REACTION[1]

V. W. ADAMKIEWICZ AND Y. LANGLOIS[2]

Abstract

A single subcutaneous injection of crystalline insulin (20 units) sensitizes considerably the rats to the dextran "anaphylactoid" reaction. This is a type of acute serous inflammation. Insulin precipitates the reaction after a very small dose of dextran (0.05–0.01 ml., 6% solution) has been injected into a "shock organ". Without insulin, such small doses of dextran are quite ineffective. Insulin also precipitates the reaction when dextran (1.0 ml., 6% solution) is injected peripherally on the back. In this site and at this dose, it rarely produces the reaction in normal rats. The sensitization manifests itself despite a cortisone pretreatment.

Introduction

A single intraperitoneal or intravenous injection of dextran (1.0 ml., 6% solution) to rats produces an acute serous inflammation of the type named "anaphylactoid" reaction (4). This inflammation is characterized by conspicuous swelling, edema and hyperemia of the paws, ears, snout, clitoris, penis, scrotum, and the anal region. The affected areas have been named "shock organs" (5). If dextran is injected into one of the "shock organs", it induces either the same type of general "anaphylactoid" reaction, or merely a local one, depending on the dose.

We report that insulin sensitizes the rat considerably to this reaction.

Methods

Male Sprague–Dawley rats (120–150 g.) were used. They were maintained on Purina Fox Chow cubes and water. Twenty-four hours before the beginning of experiments, water was substituted by a 5% w/v solution of glucose.

A 6% solution, w/v, of dextran in saline (Abbott) was used. In Experiments I and III, this was injected subcutaneously with a No. 27 needle and a tuberculin syringe into the plantar region of the right hind paw. After injection, a brief massaging of the injected area was performed. In Experiment I, a dose of 0.05 ml. was administered; in Experiment III the dose was 0.2 ml. In Experiment II, dextran was injected subcutaneously on the back, at the dose of 1.0 ml.

Those rats which were to receive insulin had 20 units of a crystalline insulin in saline (Insulin Toronto, Connaught) injected subcutaneously on the back, simultaneously with the injection of dextran, but at some distance from it.

Rats pretreated with cortisone were injected subcutaneously, on the back, with 6 mg. of cortisone acetate (Cortone, Merck) per rat, per day, during 4 days. The animals from this experiment were weighed daily.

[1]Manuscript received November 6, 1956.
Contribution from the Département de Physiologie, Université de Montréal, Montreal, Quebec.
[2]Fellow of the Lederle Medical Students' Research Fellowship.

The extent of the general "anaphylactoid" inflammation was measured by the swelling of the left hind paw. In Experiments I and III this was the paw which was not injected with dextran. The swelling was measured using a volume displacement apparatus described previously (1) and was expressed in milliliters. The volume of the paw was first determined prior to the injection of dextran and then at intervals of about 30 minutes, and 1, 2, 3, and 4 hours after the swelling. The increase in the volume of the paw after swelling was taken as the measure of the inflammatory general "anaphylactoid" reaction.

The results were analyzed statistically (2, 3).

Results

(I) Sensitization by Insulin to a Small, Ineffective Dose of Dextran in Normal Rats

Twenty rats were divided into two groups of 10. Group I were the controls. They were injected in the right hind paw with 0.05 ml. of dextran—a small dose, ineffective to produce a general "anaphylactoid" reaction. Group II received the same small dose plus a simultaneous injection of crystalline insulin.

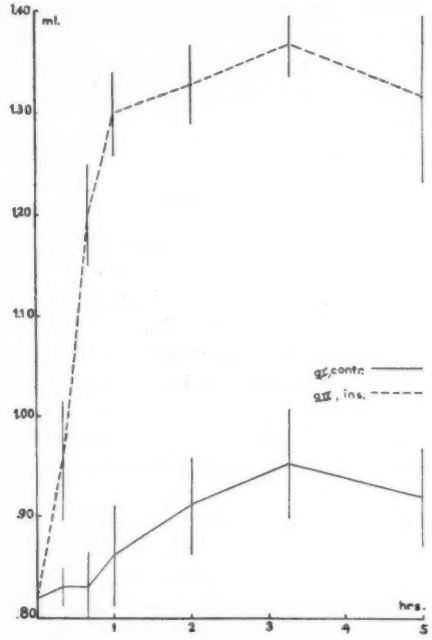

FIG. 1. Sensitization by insulin to the "anaphylactoid" reaction following the injection of dextran into a "shock organ". Abscissa: hours after injection. Ordinate: volume in milliliters of non-injected left hind paw. Group I: rats injected with an insufficient dose of dextran into right hind paw. The swelling of the non-injected left hind paw (general "anaphylactoid" reaction) is negligibly small. Group II: rats similarly injected with dextran plus one subcutaneous injection of 20 units insulin. Swelling of non-injected left hind paw is now very intense. (Vertical lines = standard error × 2.)

In the control Group I, the swelling of the non-injected, left hind paw was negligible, as expected. However, in the rats of Group II, the onset of swelling was rapid and its volume considerable. The volume of the paws before swelling was 0.82 ml. ± S.E. 0.02, and after swelling 1.36 ml. ± S.E. 0.04—an increase of 0.54 ml.

Fig. 1 represents the volumes of these paws, and the standard errors. The difference between the curves of swelling in Groups I and II is statistically highly significant throughout.

(II) Sensitization by Insulin to Dextran Injected Peripherally into Non-shock Areas

Twenty-four rats were divided into two groups. Group I, 11 rats, were the controls. These rats received subcutaneously on the back 1.0 ml. of dextran. At this peripheral site, such a dose of dextran rarely produces the "anaphylactoid" reaction. Group II, 13 rats, received a similar injection of dextran, plus a simultaneous injection of insulin.

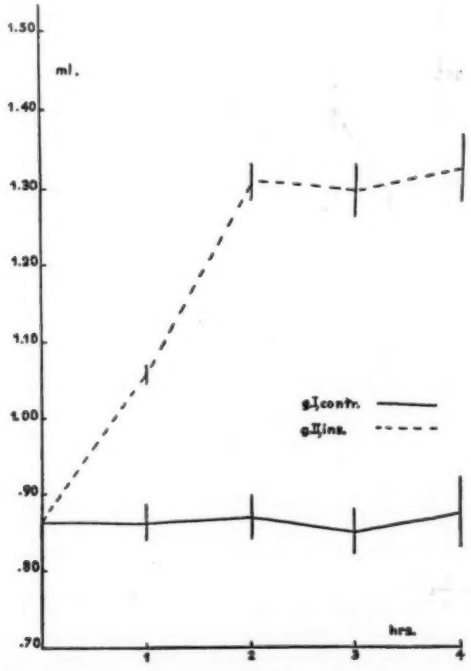

FIG. 2. Sensitization by insulin to the "anaphylactoid" reaction following injection of dextran into "non-shock areas". Abscissa: hours after injection. Ordinate: volume in milliliters of the left hind paw. Group I: rats injected subcutaneously on the back with a large dose of dextran. The left hind paw did not swell. Group II: rats similarly injected with dextran plus 20 units insulin. Swelling of the left hind paw (general "anaphylactoid" reaction) is very intense. (Vertical lines = standard error × 2.)

As expected, not one rat of Group I showed signs of the general "anaphylactoid" reaction. In Group II, all the rats reacted very intensely. The results are shown on Fig. 2. The difference between the two curves of swelling is of course highly significant.

It may be noted incidently that the curve of the volumes of paws of Group I is almost a straight line since the rats did not react and their paws did not swell. These paws were measured five times during the 4 hour period. Each time the result was practically the same. This illustrates the fair exactitude of the volumetric method applied.

(III) Anticortisone Effect of Insulin on the Dextran "Anaphylactoid" Reaction

Twenty-six rats were divided into three groups. Group I, eight rats, were the absolute controls. Group II, again eight rats, were the cortisone controls. Group III, 10 rats, received cortisone and insulin. A dose of 0.2 ml. of

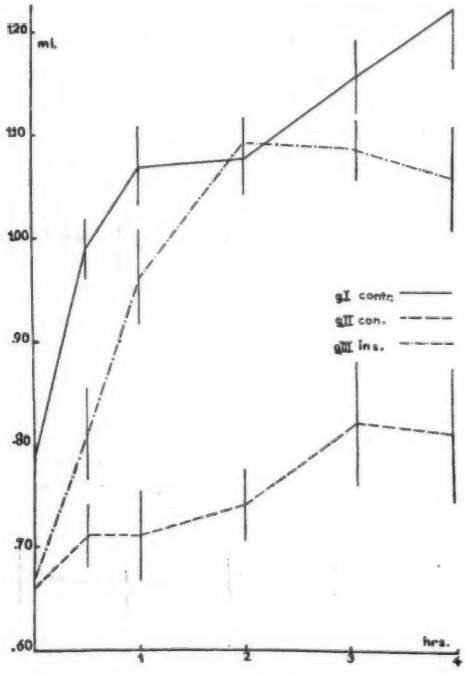

FIG. 3. Anticortisone effect of insulin on dextran "anaphylactoid" reaction. Abscissa: hours after dextran injection. Ordinate: volume in milliliters of left hind paw. Group I: rats injected with an effective dose of dextran into right hind paw. The swelling of the left hind non-injected paw (general "anaphylactoid" reaction) follows its normal intense course. Group II: rats similarly injected with dextran but pretreated 4 days with 6 mg. cortisone. The intensity of swelling of left paw is diminished and delayed. Group III: rats which were similarly injected with dextran and pretreated with cortisone, but which received 20 units insulin subcutaneously. The intensity and onset of "anaphylactoid" reaction is restored to normal. (Vertical lines = standard error × 2.)

dextran was injected into the right hind paw of rats in Groups I, II, and III. This dose, injected into the "shock organ", is sufficient to produce a general "anaphylactoid" reaction. Cortisone was administered as pretreatment for 4 days to Groups II and III. Insulin was injected simultaneously with dextran to Group III.

The body weight of cortisone-treated rats, Groups II and III, remained stationary during the pretreatment period (initial: 130 g.; final: 128 g.). The absolute controls, Group I, gained 25 g. during the same time (initial: 130 g.; final: 155 g.).

The intensity of swelling in the three groups is shown on Fig. 3. The absolute control rats, Group I, showed the normal high degree of general "anaphylactoid" swelling as measured on the left hind non-injected paw. In the rats of Group II, the pretreatment with cortisone diminished the reaction very considerably, as expected (Group I/Group II, $p < 0.01$). However, in Group III where insulin was given, the reaction was again intense, despite the cortisone pretreatment. It was statistically comparable to the reaction in normal rats of Group I (Group I/Group II, $p > 0.90$), and it was much more intense than in the cortisone group, Group II (Group II/Group III, $p < 0.01$).

Discussion

The polysaccharide dextran is one of a group of chemically different substances all of which elicit the "anaphylactoid" reaction (5). The reaction has often been used as an experimental model of a typical acute inflammation.

Insulin sensitizes the rat to the dextran "anaphylactoid" reaction. The sensitization can be obtained by injection of insulin–zinc–protamine. It can also be obtained by injection of crystalline insulin. Therefore, it is not due to some effects of protamine. Crystalline insulin contains traces of zinc. However, the sensitization does not appear to be due to the zinc, since the injection of an equivalent amount of zinc acetate does not cause sensitization. Thus, the sensitization is an effect either of the crystalline insulin itself, or of some unknown contaminant in it. In this latter case, the contaminant would have to be an extremely potent sensitization factor of this particular reaction, since the amounts of it present in the dose of injected insulin would be very small.

It is not yet known whether the sensitization by insulin to "anaphylactoid" reaction is specific for dextran or not. However, in the present case the final over-all effect of insulin is to allow more fluid to pass out of the capillaries of the "shock organs".

How sensitization by crystalline insulin is brought about remains a matter for speculation. The effect of a hypoglycemia cannot yet be excluded, although the rats were fed adequately and received a 5% solution of glucose to drink during the 24 hours preceding the end of each experiment. In a recent experiment (unpublished) sensitization was obtained with a dose of 0.1 units insulin–zinc per 100 g. body weight in the rat, in a setup similar to Experiment II.

A 4 day pretreatment with 6 mg. of cortisone per rat diminishes considerably the intensity of the dextran "anaphylactoid" reaction, and retards its onset by 1 to 2 hours. Six milligrams of cortisone is a maximum dosage. In fact, this amount is too large to be absorbed from the injection site. Such pretreatment induces marked histological changes. Despite this, a single administration of insulin to cortisone-pretreated rats restores the rapid onset and the intensity of the reaction. (However, the intensity does tend to diminish in this case more rapidly than in the normal animal.) The sensitization by insulin is therefore a very rapid process. It manifests itself within minutes after injection.

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THE NATURE OF THE SUBSTANCES IN DIETARY FAT AFFECTING THE LEVEL OF PLASMA CHOLESTEROL IN HUMANS[1]

J. M. R. Beveridge, W. F. Connell, and G. A. Mayer

Abstract

Three dietary experiments have been performed in which 52, 48, and 38 male students and a few members of staff participated as experimental subjects. In the first experiment 52 subjects ingested for 8 days a diet in which butter provided 60% of calories. During the next 8 days subgroups were given rations supplemented with α-tocopherol or β-sitosterol, substances known to be present in corn oil in significant amounts. The α-tocopherol had no effect but the β-sitosterol caused a highly significant decrease in plasma cholesterol.

The same conditions were used for the first 8 days of the second experiment in which 48 subjects participated. The subgroups were given diets in which 30% of calories were supplied by butter and 30% by various corn oil fractions obtained by vacuum distillation. All groups showed highly significant decreases in plasma cholesterol and there did not appear to be any effective fractionation of the plasma cholesterol depressant factor in the corn oil, although the largest drop was obtained in the case of the most volatile fraction derived from the corn oil. This preparation contained most of the unsaponifiable material and had a slightly lower iodine number than the other fractions.

In the third experiment, 38 subjects ingested for 8 days a diet providing 60% of calories from corn oil. Subgroups were transferred to diets supplying 40% of calories from butter fractions obtained by vacuum distillation. All groups showed highly significant increases, the greatest increase being noted in the case of the most volatile fraction, which contained most of the unsaponifiable material. It has been postulated that the potent plasma cholesterol elevating action of butterfat is at least partly dependent upon the presence of the unsaponifiable fraction. The identity of this factor and whether it acts alone or in conjunction with certain types of fatty acid residues are questions that remain to be answered.

A few years ago during the course of studies on dietary factors affecting the level of plasma lipids in humans, we decided that definitive data could not be obtained unless better controlled experiments could be carried out. To this end homogeneous rations were devised, containing relatively few ingredients of known composition, which could be accurately made up and dispensed. Previous publications by us (4, 5, 6, 7) indicated that the ingestion of such diets containing certain fats of animal origin led to higher levels of plasma cholesterol than when these fats were replaced by equicaloric amounts of corn oil. Other investigators (1, 12) utilizing somewhat different dietary regimens and experimental designs have obtained similar results. Since we also showed that rations providing 60% of calories in the form of corn oil actually led to lower lipid levels than did diets essentially free from fat, it was concluded that the effect of this fat was due in part at least to the presence of a plasma cholesterol depressant factor and was not due solely to the elimination of some animal fat from the diet (5, 6). Thus the problem with which we were faced was the fractionation of butter, the most potent of the animal fats tested,

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and of corn oil in order to gain some idea of the nature of the plasma cholesterol elevating and depressant factors contained in these foods. The following experiments, which were performed with the co-operation of over a hundred male volunteer medical students together with a few students in biochemistry and members of staff, were carried out in an attempt to obtain further information on the nature of these unknown factors.

Experimental

The methods used in preparing and storing the diets together with the experimental design and analytical techniques have been described in previous publications (6, 7). In brief summary, the dietary ingredients were mixed together with water and the mixture thoroughly homogenized. Protein supplied 16.9% of calories in all experimental diets. The only other items permitted were water, clear tea, and clear coffee. In the course of the last two experiments reported here there were a total of three instances in which minor deviations from this strict regimen occurred. These deviations were as follows: one subject consumed at a banquet 2 sq. cm. of steak, ½ tsp. of peas and corn, and 2 tsp. of grapefruit juice; another drank one glass of grape juice; and finally another had 2 oz. of sherry. Since it was believed that these defections were minor and would not affect significantly the outcome of the results, the values from these subjects have been retained. A very small number of participants were unable to stay on the diet and for various reasons dropped out. Only a few individuals gained or lost more than 2 or 3 lb. during the experimental period and the great majority were highly successful in maintaining their body weight essentially constant. Blood samples were taken from the subjects in the fasting state between 7.00 and 8.30 a.m. and plasma cholesterol determined by a modification of the Schoenheimer–Sperry technique (17). Three large-scale experiments were performed and these are described under the headings, Experiment I, II, and III.

Experiment I

Because of certain practical considerations it was decided to test first of all the effects of two substances, β-sitosterol and α-tocopherol, both of which occur in corn oil in significant amounts. Considerable interest was aroused in the plasma cholesterol depressant effect of plant sterols following the initial report by Peterson (13) in 1951 that these compounds were effective in inhibiting the usual increase in plasma cholesterol in chicks fed a diet high in this lipid. Subsequently it was shown by Pollak (15) that a hypocholesterolemic response could be obtained in man, particularly in certain cases of hypercholesterolemia, by the administration of these substances. This investigator also indicated that there was no significant effect in subjects with cholesterol concentrations of less than 200 mg. per 100 ml. of serum. Since these reports were published, most investigators who have tested the effect of mixed plant sterols or of β-sitosterol have found decreases in some

normo- and some hyper-cholesterolemic individuals (2, 3, 10, 16). Wilkinson *et al.* (18), however, were unable to demonstrate any effect of sitosterols during a study of 35 weeks' duration. In the light of Pollak's report (15), it may be of interest to point out that three of the four patients in their study had cholesterol values less than 200 mg. per 100 ml. of serum.

Fifty-two healthy male subjects ingested a diet whose composition is shown in Table I and which supplied 60% of calories in the form of butterfat. The subjects were then divided into five groups, one of which was continued for a further 8 days on the diet high in butterfat and the others were transferred to diets which were modified by the addition of the following supplements per 950 cal. portion: 10 mg. α-tocopherol (E-10); 200 mg. α-tocopherol (E-200); 7 g. β-sitosterol (S). One group was transferred to a diet providing 60% of calories in the form of corn oil in place of butterfat.

TABLE I

BASAL DIET FOR EXPERIMENT I*
AMOUNTS REQUIRED TO MAKE A 950 CAL. SAMPLE

Ingredient	Amt. (g.)	Protein (g.)	Fat (g.)	CHO (g.)
Lesofac	60.0	30.0	0.6	23.5
Starlac	28.1	10.0	0.1	14.6
Sweet butter	77.4	0.46	62.6	0.3
Sucrose	17.1	—	—	17.1
Total		40.46	63.3	55.5
Calories	953.5	161.84	569.7	222.0
% calories		16.96	59.72	23.32

*Two grams iodized salt were added per 950 cal. batch. A mixture of vitamins was also added to supply the following amounts of these substances per 950 cal.: thiamine, 0.6 mg.; riboflavin, 0.6 mg.; niacin, 5.0 mg.; pyridoxine, 5.0 mg.; calcium pantothenate, 5.0 mg.; ascorbic acid, 25 mg. In the case of any diet that did not contain butterfat, 1700 I.U. of vitamin A were added per 950 cal. portion.

Results and Discussion

The results, shown in Fig. 1, reveal that α-tocopherol had no effect whatsoever at either level fed whereas the sitosterol brought about a highly significant decrease of 21.2% in plasma cholesterol. Those transferred to the diet high in corn oil showed a similar decrease amounting to 23.1%. These responses, expressed in terms of percentage change from the values noted at day 8, are illustrated in Fig. 2. The remarkable uniformity of the individual responses to the ingestion of β-sitosterol is demonstrated in Figs. 3 and 4.

The potent effect of this plant sterol in normocholesterolemic subjects on a diet high in butterfat was not expected on the basis of the results reported in the literature, particularly in the case of those with cholesterol values of less than 200 mg. per 100 ml. plasma. One possible reason for the uniform and significant response to the ingestion of this substance is that it was distributed uniformly throughout the entire diet. Davis (9) has pointed out that this feature is of paramount importance if cholesterol absorption is

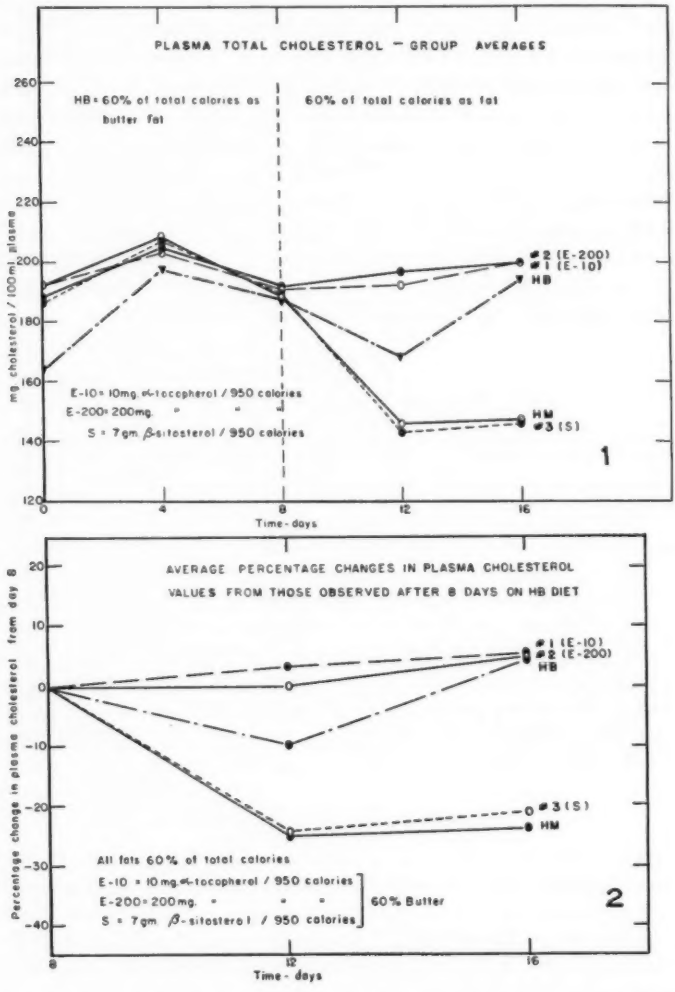


FIG. 1. Average values for plasma cholesterol of 46 subjects during a period of 8 days on a homogeneous formula diet in which butterfat supplied 60% of the total calories and during a subsequent similar period in which the subjects were divided into five groups and placed on the following diets: basal diet (HB); basal diet plus the following supplements per 950 cal. batch: 10 mg. α-tocopherol (E-10); 200 mg. α-tocopherol (E-200); 7 g. β-sitosterol (S); one group was transferred to a diet providing 60% of calories in the form of corn oil in place of butterfat (HM).

FIG. 2. Averages of the individual percentage changes in plasma cholesterol noted between day 8 and 16 for groups of young male subjects following an initial period of 8 days on a diet providing 60% of calories in the form of butterfat and placement on diets as described in the legend for Fig. 1.

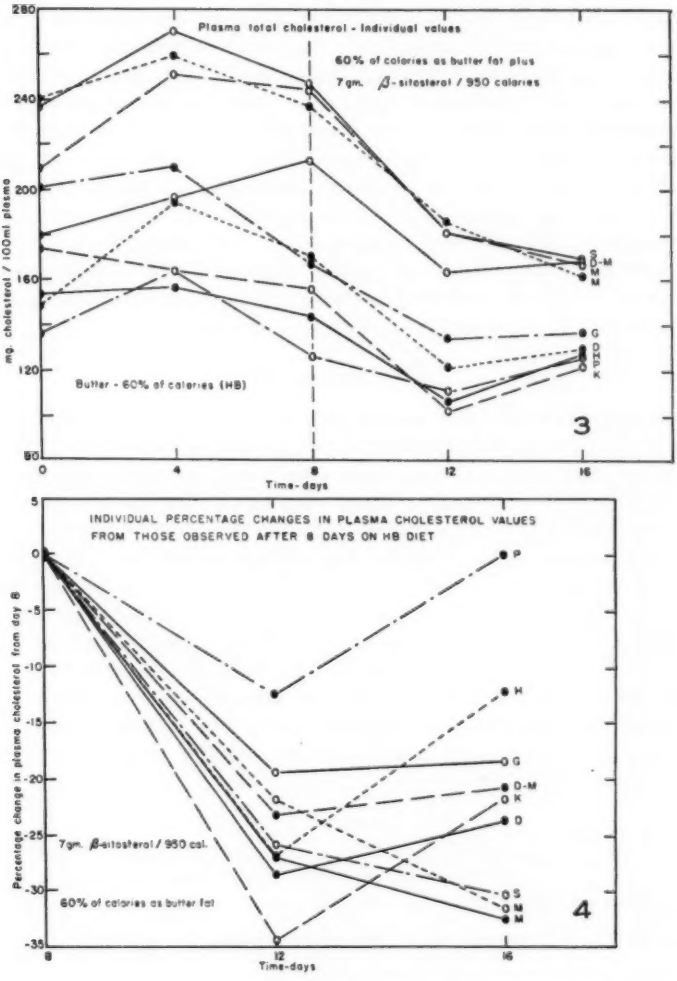


FIG. 3. Individual values for plasma cholesterol of nine subjects during a period of 8 days on a homogeneous formula diet in which butterfat supplied 60% of the total calories and during a subsequent similar period in which the subjects consumed the same diet supplemented with 7 g. β-sitosterol per 950 cal. batch.

FIG. 4. Individual percentage changes in plasma cholesterol noted between day 8 and 16 for nine subjects following an initial period of 8 days on a diet providing 60% of calories in the form of butterfat and placement on a supplemented diet containing 7 g. β-sitosterol per 950 cal. batch.

decreased by formation of a mixed crystal complex with sitosterol. The mode of administration of this sterol in the experiment reported here is to be contrasted with that described by other workers who gave it separately, usually in the form of a suspension before or during meals. Such a result naturally raises the possibility, now being tested, that the presence of this sterol in corn oil is at least in part responsible for the plasma cholesterol depressant effect of this fat. It is of interest in the light of the results reported here to refer to the work of Peterson, Nichols, Peek, and Chaikoff (14), who obtained a significant decrease of serum cholesterol in a group of subjects by mixing 1.9 g. of soy sterols with a 14 g. portion of butterfat per meal. The other possibility that has been suggested as being responsible for the plasma cholesterol depressant action of certain vegetable oils is their content of unsaturated fatty acid residues, possibly the essential fatty acids, as proposed by Kinsell *et al.* (11) and by Bronte-Stewart and his colleagues (8).

Experiment II

The next experiment was carried out to obtain further evidence on the nature of the plasma cholesterol depressant factor in corn oil. Through the kind co-operation of Drs. Philip Harris and Norris Embree of the Distillation Products Industries Limited, a large amount of this oil, procured in the U.S.A., was fractionated by a process of molecular distillation in high vacuum into four fractions. The distillate was divided as follows: Fraction No. 1 comprised the first 10% of the distillate; Fraction No. 2, the second 10% (10–20%); Fraction No. 3, the next 30% (20–50%); and Fraction No. 4, the residue (50–100%). The total unsaponifiable matter and iodine numbers of these fractions are shown in Table II. Forty-eight volunteer male subjects ingested for 8 days the same diet high in butterfat as was used during the initial phase of Experiment I. Groups were then transferred for a further 8 days to diets in which 30% of calories were supplied by butterfat and 30% by the various corn oil fractions.

TABLE II

ANALYTICAL DATA ON CORN OIL AND FRACTIONS DERIVED THEREFROM BY DISTILLATION

	1st cut (0–10%)	2nd cut (10–20%)	3rd cut (20–50%)	4th cut (50–100%)	Corn oil
Unsaponifiable	6.48	2.02	0.99	0.61	1.28
Iodine No.	113.4	116.7	117.7	122.1	122.0

Results and Discussion

The average plasma cholesterol levels are shown in Figs. 5 and 6. All fractions brought about highly significant decreases. The first 10% cut, which contained most of the unsaponifiable material and which exhibited a slightly lower iodine number than did the other fractions (cf. Table II),

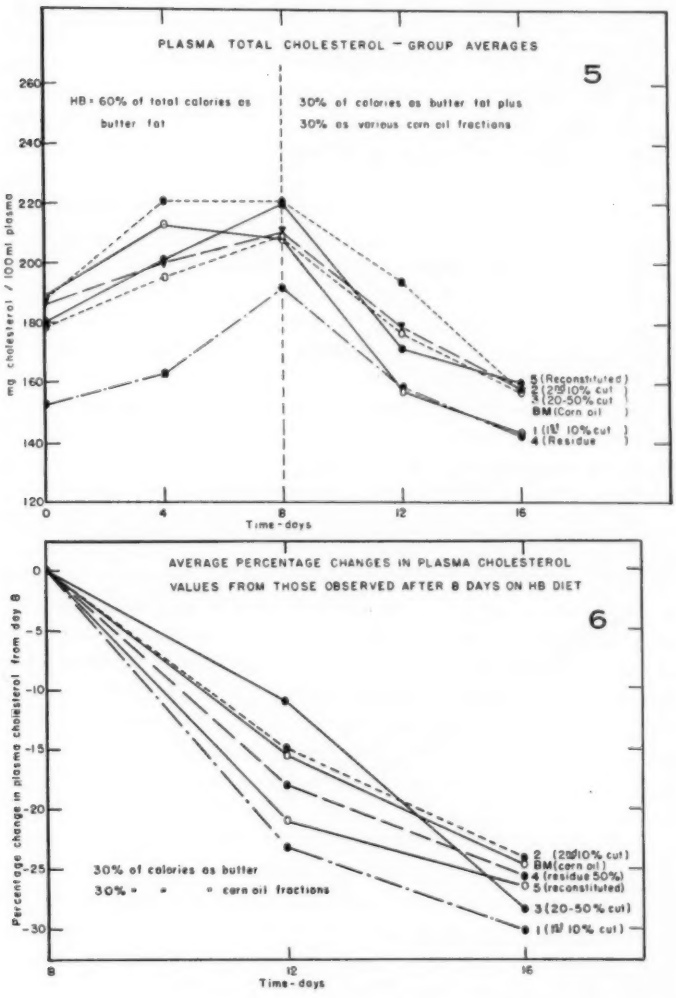
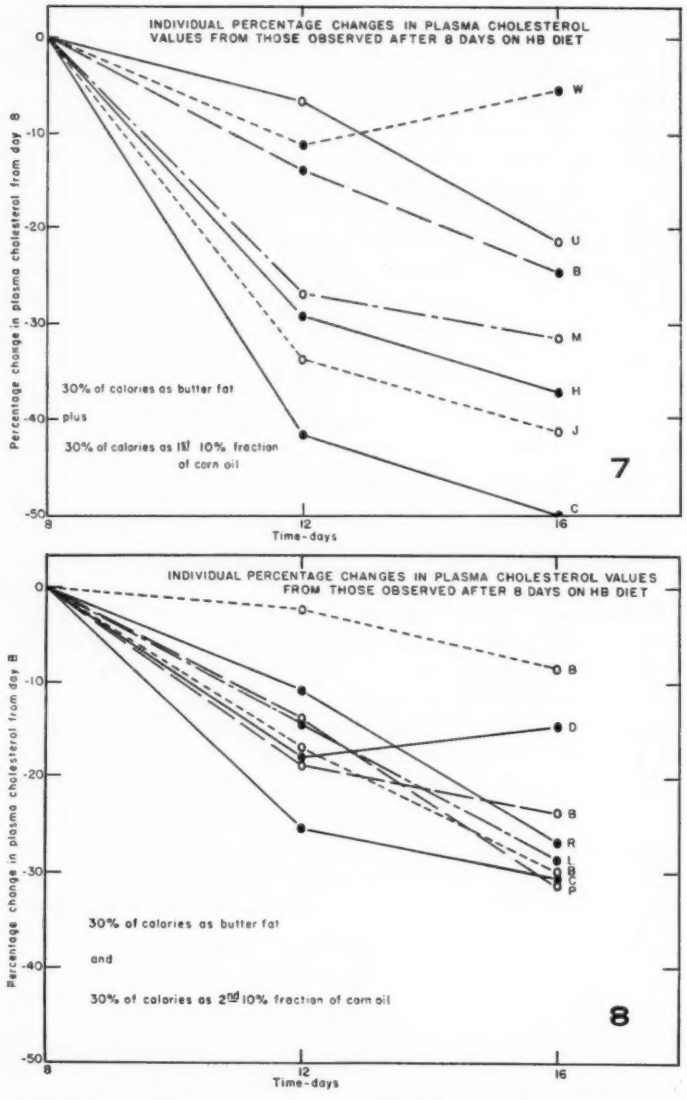


FIG. 5. Average values for plasma cholesterol of 46 subjects during a period of 8 days on a homogeneous formula diet in which butterfat supplied 60% of the total calories and during a subsequent similar period in which the subjects were divided into six groups and placed on diets modified in respect of the fat component as follows: 30% of calories from butterfat plus 30% of calories from corn oil or fractions thereof.

FIG. 6. Averages of the individual percentage changes in plasma cholesterol noted between day 8 and 16 for groups of young male subjects following an initial period of 8 days on a diet providing 60% of calories in the form of butterfat and placement on diets as described in the legend for Fig. 5.



FIGS. 7 and 8. Individual percentage changes in plasma cholesterol noted between day 8 and 16 following an initial period of 8 days on a diet providing 60% of calories in the form of butterfat and placement on a diet modified in respect of the fat component as follows: 30% of calories from butterfat plus 30% of calories from the first cut of corn oil (cf. Fig. 7) or from the second cut of corn oil (cf. Fig. 8).

effected the greatest decrease, 30.1%, but this result could not be shown to be significantly different from the responses observed with the other fractions. The second, third, and fourth cuts brought about percentage decreases of 24.2, 28.4, and 25.9 respectively. That no potent material was destroyed during the distillation may be concluded from a comparison of the effects of diet BM, which contained the original corn oil, and diet No. 5, which contained reconstituted oil prepared by combining appropriate amounts of the 1st, 2nd, 3rd, and 4th fractions. The decreases were 24.7 and 26.4% respectively.

In assessing these results it should be kept in mind that the mere elimination of butterfat from the diet would by itself cause a decrease in plasma cholesterol. The changes observed here, therefore, are due to a combination of two factors: firstly, a decrease in the dietary level of butterfat, and secondly, the addition of corn oil or fractions thereof. Although there did not appear to be any effective fractionation of the plasma cholesterol depressant factor, the differences noted between the effects of the first two cuts as demonstrated in Figs. 7 and 8 are suggestive that some fractionation did in fact occur.

In retrospect it would appear that the conditions selected for assessing the plasma cholesterol depressant effect of the corn oil fractions were not ideal since even the diet providing 30% of calories as butterfat and 30% as corn oil led to a much greater fall than was expected on the basis of past experience, and indeed this decrease approached the order of magnitude expected for a ration supplying 60% of calories as corn oil. The ratio of 30% of calories from butterfat and 30% from corn oil and fractions thereof was chosen on the basis of work published recently in which we found that such a ration produced an intermediate decrease in plasma cholesterol, the average drop being 15.9%. If such an intermediate depression had been obtained in the present experiment with the butterfat – corn oil ration (BM), differences in plasma cholesterol depressant properties of the fractions would have been much more readily detected. One rather obvious conclusion that may be drawn from this experience is that different batches of corn oil and/or butterfat vary in their quantitative effects on plasma cholesterol levels.

Finally it may be concluded that this experiment, although providing further suggestive evidence that the plasma cholesterol depressant effect of corn oil, and presumably certain other vegetable oils, is dependent at least in part upon the composition of the unsaponifiable matter; there is no doubt whatsoever that further work is required to establish the relative importance and role of this material and that of the unsaturated fatty acid residues.

Experiment III

In this experiment an effort was made to gain some idea of the cholesterol elevating factor in butterfat by testing various butter oil fractions obtained by the process of molecular distillation from the same source mentioned previously. Thirty-eight subjects ingested for 8 days a diet similar to that

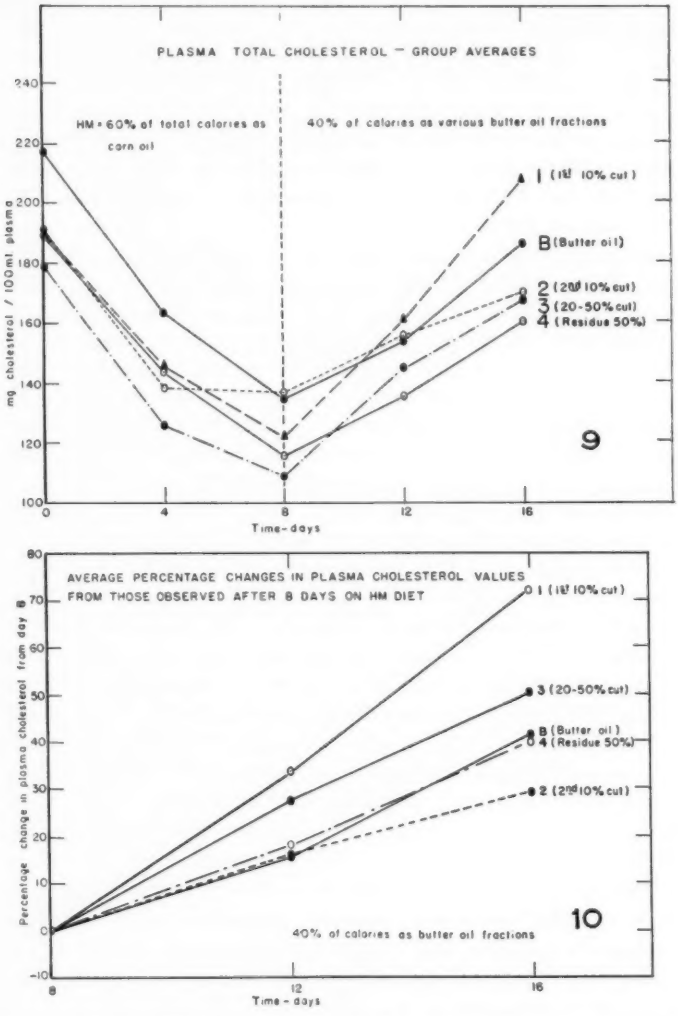


FIG. 9. Average values for plasma cholesterol of 34 subjects during a period of 8 days on a homogeneous formula diet in which corn oil supplied 60% of the total calories and during a subsequent period in which the subjects were divided into five groups and placed on diets in which the fat component provided a total of 40% of calories from butter oil or fractions thereof. (Values for day 0, 4, 8, and 12 for plasma cholesterol of a subject who was on diet No. 1 only until the end of the 12th day were as follows: 185.3, 117.7, 122.0, and 175.5 mg./100 ml. respectively.)

FIG. 10. Averages of the individual percentage changes in plasma cholesterol noted between day 8 and 16 for groups of young male subjects following an initial period of 8 days on a diet providing 60% of calories in the form of corn oil and placement on diets in which the fat component provided a total of 40% of calories from butter oil or fractions thereof.

used in Experiment I except that 60% of calories were provided by corn oil in place of butterfat. Groups were then placed for a further 8 days on rations supplying 40% of calories from the butter fractions, which were comprised of Fraction No. 1, the first 10% of the distillate; Fraction No. 2, the second 10% (10–20%); Fraction No. 3, the next 30% (20–50%); and Fraction No. 4, the residue (50–100%). The total unsaponifiable matter, saponification values, and iodine numbers for these fractions are shown in Table III.

TABLE III

ANALYTICAL DATA ON BUTTER OIL AND FRACTIONS DERIVED THEREFROM BY DISTILLATION

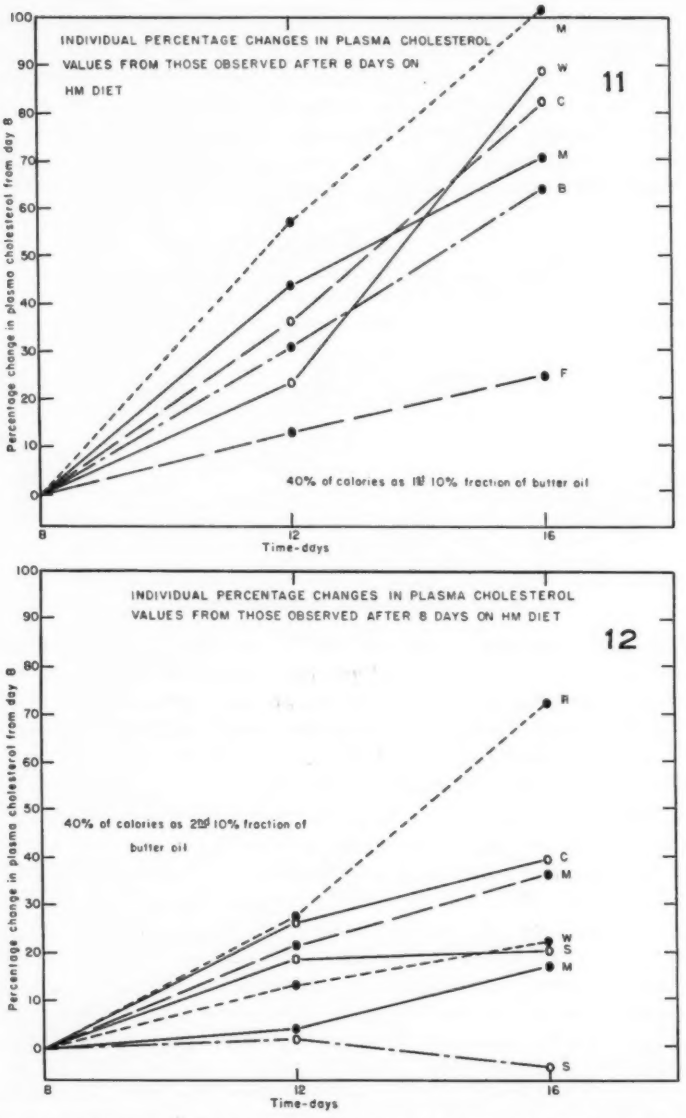
	1st cut (0–10%)	2nd cut (10–20%)	3rd cut (20–50%)	4th cut (50–100%)	Butter oil
Saponification No.	250.8	252.0	235.0	203.0	222.5
Iodine No.	24.5	26.6	34.8	48.5	39.8
Digitonin-precipitable Lieberman-Burchard positive material	1.60	0.115	Trace	Trace	0.146

Results and Discussion

The average responses shown in Fig. 9 demonstrate highly significant increases in plasma cholesterol in all groups, the greatest increase being noted in the most volatile fraction. These results are also shown in Fig. 10 expressed as average percentage changes from the values noted at day 8. The percentage increases for Fractions 1, 2, 3, and 4 and the original butter oil No. 5 are respectively: 71.7, 29.3, 50.8, 40.1, and 41.3.

Again, as was the case in the interpretation of the results of the previous experiment, attention should be drawn to the fact that these increases in plasma cholesterol are due to a combination of two factors: firstly, the elimination of the plasma cholesterol depressant factor from the diet by omission of the corn oil; and secondly, the introduction of the plasma cholesterol elevating factor in the butter oil and butter oil fractions. It follows from this consideration and from our previous work (5, 6,) that transfer from the diet containing corn oil which was ingested during the first 8 days to a diet free from fat would have resulted in a significant increase. However, there would appear to be a good possibility that Fractions 2, 3, and 4 caused elevations of plasma cholesterol greater than those that would have occurred on transfer from a diet containing corn oil to a fat-free diet. This may simply be due to a non-specific effect of triglycerides in general in the absence of any plasma cholesterol depressant factor such as is present in corn oil.

A comparison of the individual responses to the ingestion of diets containing Fractions No. 1 and No. 2 (cf. Figs. 11 and 12) certainly indicates that a concentration of the plasma cholesterol elevating material present in butter



FIGS. 11 and 12. Individual percentage changes in plasma cholesterol noted between day 8 and 16 following an initial period of 8 days on a diet providing 60% of calories in the form of corn oil and placement on diets in which the fat component provided a total of 40% of calories from the first cut of butter oil (cf. Fig. 11) or from the second cut of butter oil (cf. Fig. 12). One subject on the diet containing the first cut of butter oil stayed on this ration only until the end of the 12th day and it is of interest to note that the percentage increase for this individual at this point (43.9) was higher than the average for the rest of the group (34.0) and of course much higher than that of the group on the diet containing the second cut of butter oil (16.2).

had been made in the first 10% cut. It must be pointed out, however, that the difference in the average responses could not be shown to be significant*.

Certain analytical data obtained on the butter oil and fractions thereof are shown in Table III. In comparing the composition of the first two cuts the only obvious difference lies in amounts of the total unsaponifiable and the digitonin-precipitable, Lieberman-Burchard positive material. It would appear reasonable to suggest that the plasma cholesterol elevating factor of butterfat is present in or dependent upon the presence of the unsaponifiable fraction. The identity of this factor and whether it acts alone or in conjunction with certain fatty acid residues or triglycerides are questions that remain to be answered. Further fractionations of these butter oil samples are currently being carried out with a view to gaining information on these problems.

Acknowledgments

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The authors are indebted to John Wyeth and Brother (Canada) Ltd. for generous supplies of certain of the dietary ingredients.

Through the kind co-operation of Drs. Philip Harris and Norris Embree of the Distillation Products Industries Limited, Division of Eastman Kodak Company, supplies of fractionated fats and α-tocopherol were made available and we thank them for this. One of us has also had the benefit of several helpful consultations with these and other members of the research staff of this organization.

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*Of interest in this connection is a personal communication to one of us (J.M.R.B.) from Dr. E. H. Ahrens of the Rockefeller Institute for Medical Research indicating that he had previously performed somewhat similar experiments on two patients using two cuts, 0–50 and 50–100%, from corn oil and the same cuts from lard. He was unable to show that these two pairs of fat fractions produced a significant difference in serum lipid levels.

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NOTES

PAPER ELECTROPHORETIC SEPARATION OF WATER-SOLUBLE CASTOR BEAN PROTEINS[1]

P. A. Adie and A. D. Robinson

Proteins identified in castor beans and castor bean extracts include albumin (3) and globulin (4). The most extensive studies are those of Osborne, Mendel, and Harris (2), who believe that there are present one albumin, one globulin, and several proteoses. In these investigations the classical techniques of extraction, dialysis, and precipitation were used. An examination of water extracts of oil-free castor bean meal, made by us by paper electrophoresis, has revealed the presence of all of these fractions.

Experimental

Oil was removed from ground castor beans with cold ether and the oil-free residue was extracted with distilled water, the proportions being 25 g. of meal to 100 ml. of water. The extract was subjected to paper electrophoresis in an apparatus similar to that employed by Cremer and Tiselius (1) except that carbon electrodes were employed in electrode vessels joined to the rest of the assembly with filter-paper wicks saturated with buffer solution. Schleicher and Schüll paper 598*g* and a buffer solution at pH 6.0—made by mixing 0.2 molar (*M*) acid potassium phosphate and 0.2 *M* sodium hydroxide —were found most satisfactory. A potential of 140 v. was applied for 48 hours.

The protein was fixed in the paper by drying and heating at 100° C. for 20 minutes. It was dyed with azocarmine B solution according to the procedure of Turba and Enenkel (5). Five colored areas appeared, indicating that there were five protein fractions in the extract. There was, as well, a dark mark at the starting point, due to some nonmobile material whose identity was not established. The filter paper was cut perpendicularly to the flow of current into strips 0.5 cm. wide. The dye was eluted from each small strip with 5 ml. 10% sodium carbonate in 50% ethanol. The transmittance of each extract was determined with a Coleman spectrophotometer, Model 11, at 498 mμ. The data of these measurements are reproduced in graphical form in Fig. 1. A peak at *S* is due to the nonmobile material referred to above. The other five peaks represent the five mobile fractions and are designated by the letters *A* to *E*.

[1]This work was condensed from a thesis submitted to the Faculty of Graduate Studies and Research of the University of Manitoba in partial fulfillment of the requirements for the degree of Master of Science, by P.A. Adie, May, 1952.

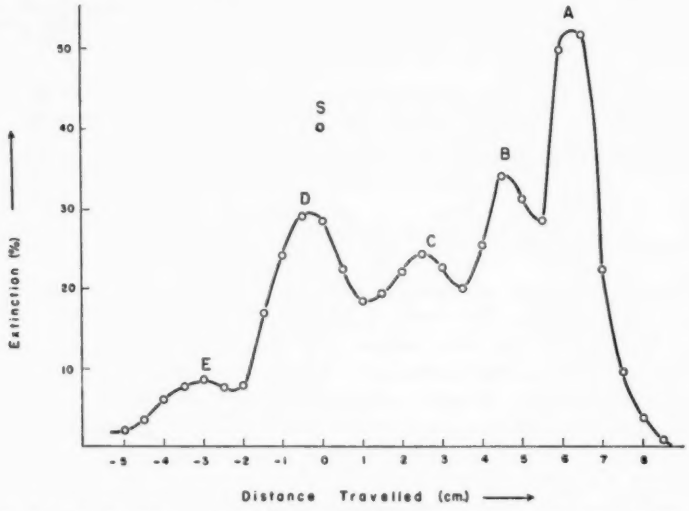

FIG. 1. Per cent extinction and distance travelled towards the cathode by protein fractions.

These fractions were identified as follows: The original protein extract was dialyzed against distilled water. A precipitate formed. This was soluble in 10% sodium chloride, indicating it was a globulin. A portion of the precipitate was dissolved in phosphate buffer solution at pH 6.0 and subjected to the usual electrophoresis and processing. Only the *A* fraction was revealed by the resulting data and graphs, indicating that the *A* fraction was a globulin. The rest of the dialyzate was heated at 70° C. for 4 hours and the precipitate which formed, an albumin, was separated by filtering. This temperature was selected as the lowest which would bring about coagulation of the albumin and not harm other proteins present. It was used by Osborne *et al.* (2) for the same purpose. The filtrate was subjected to the usual electrophoretic separation and processing. It was found that fraction *E* had been eliminated. Therefore fraction *E* was an albumin, and *B*, *C*, and *D*—which were soluble in water and not coagulated by heat—are described as proteoses.

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